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Study on Complex Formation Between Recombinant Human Thrombomodulin Fragment and Thrombin Using Surface Plasmon Resonance

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Human thrombomodulin (hTM) is a newly described endothelial cell associated protein that functions as a potent natural anticoagulant by converting thrombin from a procoagulant protease to an anticoagulant. The affinity constant of recombinant human soluble TM (rhs-TM) and a peptide containing active site of hTM fragment (f-hTM) with thrombin were determined using the surface plasmon resonance. The interaction of f-hTM with thrombin could be analyzed by a simple model, whereas the association and the dissociation steps of rhs-TM with thrombin consisted of at least two kinds of interaction phases. The dissociation constant for complex (K_D) of f-hTM and thrombin was determined to be 205 nM, which was more than twice as high as that of rhs-TM (6.7 and 75 nM). The lower affinity of f-hTM was not due to the slow association rate but to the rapid dissociation rate. It comes clear that f-hTM interacts with thrombin rapidly. Am. J. Hematol. 63:136–140, 2000.

Key words: surface plasmon resonance; thrombomodulin; thrombin; binding constant

INTRODUCTION

Many studies have been done on the preparation of the antithrombogenic materials and on the development of artificial internal organs [1]. One of the most practical methods is the incorporation of biologically active substances which have antithrombogenic activity, such as heparin [2–5]. Human thrombomodulin (hTM), a recently discovered endothelial cell associated protein that functions as a potent natural anticoagulant by converting thrombin from a procoagulant protease to an anticoagulant [6–11], can also be used to develop antithrombogenic biomaterials. We have performed the immobilization of hTM onto various substrates using recombinant human soluble hTM (rhs-TM) [12–16] and found that rhs-TM-immobilized materials showed excellent antithrombogenicity. rhs-TM is one of the most valuable biologically active substances that can endow antithrombogenicity on the surface of a polymer.

hTM has been studied extensively in order to find its active antithrombogenicity site (in particular, in regard to its binding to thrombin), in the 4th, 5th, and 6th endothelial growth factor-like domains (EGF-456 domain). Using protein engineering or genetic engineering technologies, researchers have synthesized and studied various hTM-related peptides [17–19]. Recently, a novel peptide that contained an active hTM sequence (EGF-456 domain) and sugar moiety was synthesized (recombinant h-TM fragment: f-hTM) [20]. In previous research, the antithrombogenic activity of f-hTM was shown, and f-hTM has been analyzed as an anticoagulant drug or antithrombogenic modifier of biomaterials [21]. The antithrombogenic activity of hTM is known to have

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at least two mechanisms: the entrapment of thrombin and the activation of protein C via the hTM-thrombin complex. The interaction of hTM and thrombin is essential in both of them, therefore, research on the affinity of the newly synthesized f-hTM to thrombin is important in the development of a novel antithrombogenic biomaterial that is incorporated with f-hTM.

In this study, using surface plasmon resonance (SPR), the kinetic constants of f-hTM and thrombin were determined and compared with those of rhs-hTM. SPR is useful in studying the interactions between two kinds of materials, especially for studying rapid reactions and weak interactions with a very small amount of reagents.

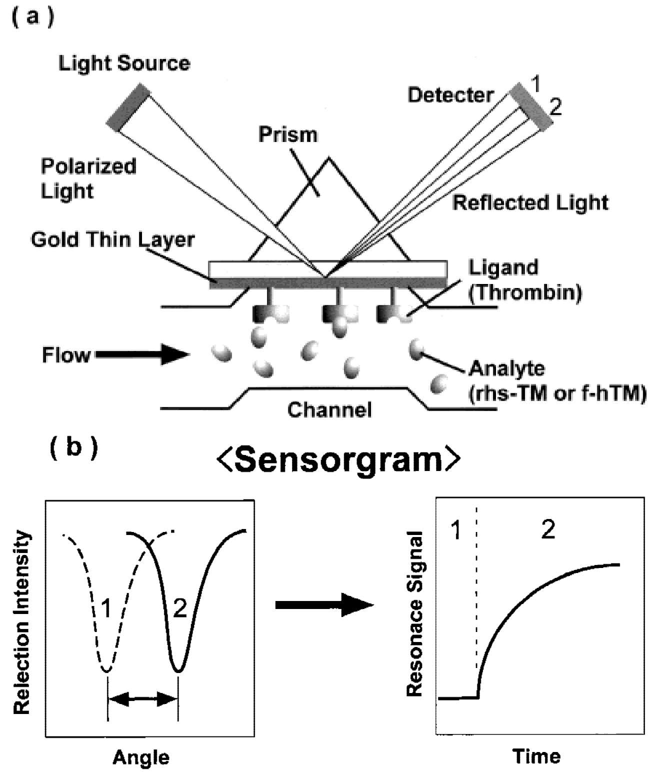

Fig. 1. SPR detection system. (a) Schematic representation of sensor chip and detecting system. (b) Example of sensorgram.

MATERIALS AND METHODS

Materials

Recombinant f-hTM (lot no. E05A5L) and rhs-TM (lot no. M173KII, Type II thrombomodulin) were synthesized and donated by the Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). The purity of both f-hTM and rhs-TM were over 99.5%. The molecular masses of f-hTM and rhs-TM were 17 kDa (114 amino acids and polysaccharide) and 71 kDa (498 amino acids and polysaccharide), respectively [20]. Thrombin was purchased from Sigma (St. Louis, MO). Other chemicals were purchased from WAKO Pure Chemicals (Osaka, Japan).

SPR Study

The interaction between f-hTM or rhs-TM and thrombin was monitored by SPR detection using a BIAcore 2000 (Pharmacia Biosensor AB, Uppsala, Sweden). Thrombin was covalently attached to the carboxymethylated dextran matrix of a sensor chip (CM-5 chip: research grade, Pharmacia Biosensor AB). After the derivatization step, any non-covalently bound thrombin was removed from the surface of sensor using 0.1 M PBS for 3 min. The binding of f-hTM or rhs-TM to the immobilized thrombin was monitored by passing aliquots (40 μl) of the f-hTM of rhs-TM (from 10 to 600 nM) in a buffer (0.01 M Tris-HCl, pH 7.4, that contained 0.1 M NaCl, 5 mM $CaCl_2$) across a sensor plate at a flow rate of 10 μl/min (Fig. 1a). SPR occurs when surface plasmon waves are excited at the metal/liquid interface. Binding between thrombin and rhs-TM/f-hTM causes changes in the refractive index at the surface layer, and these are detected as changes in the SPR signal. This change is measured continuously to form a sensorgram, which provides a complete record of the progress of association or dissociation (Fig. 1b). The sensorgram of the binding step was recorded for 240 sec, and then the buffer was injected in order to analyze the dissociation step. Between assays, the surface was regenerated by washing the surface of the sensor with 1.5 M NaCl for 3 min.

Kinetic Analysis

The reaction between the immobilized thrombin and the f-hTM or rhs-TM (analytes) can be assumed to follow pseudo-first-order kinetics. The association rate constant (k_a) and the dissociation rate constant (k_d) were calculated by nonlinear regression analyses using a BIAcore 2000 program (BIAevaluation ver. 3.0). The dissociation constant for the complex (K_D) was calculated as $K_D = k_d/k_a$. The evaluation model and kinetic equations are described in references [22,23].

RESULTS

Thrombin was covalently coupled to the dextran surface of the BIAcore sensor chip using primary amine groups (Fig. 2). Prior to the experiment of the interaction of f-hTM or rhs-TM and thrombin, the nonspecific interaction of f-hTM or rhs-TM and the surface of the sensor chip (CM-5) were investigated to check the background of the sensorgram. No binding of f-hTM to the surface of CM-5 chip was detected. On the other hand, rhs-TM showed nonspecific binding to the same surface. In order to normalize the nonspecific interaction of rhs-

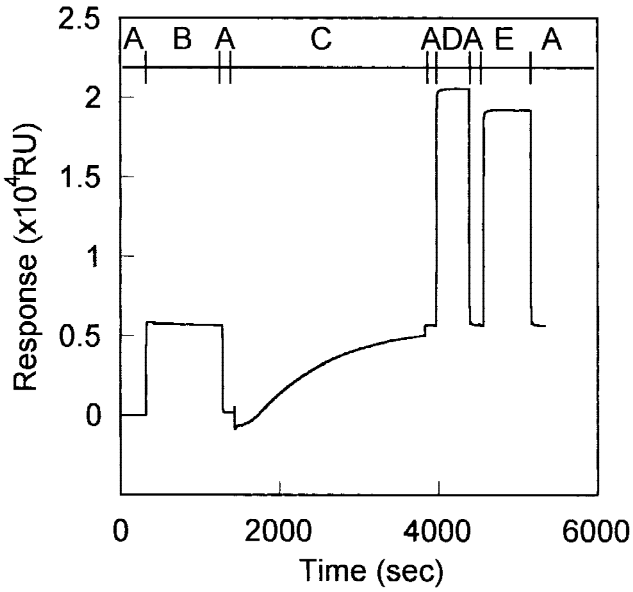

Fig. 2. Sensorgram for immobilization of thrombin on to the sensor chip. The letters indicate the time intervals during which the sensor chip is in contact with different solutions. (A) PBS; (B) cross-linking agent; (C) thrombin solution in saline sodium citrate buffer (pH 6.5); (D) ethanolamine hydrochloride; (E) 1.5 M NaCl.

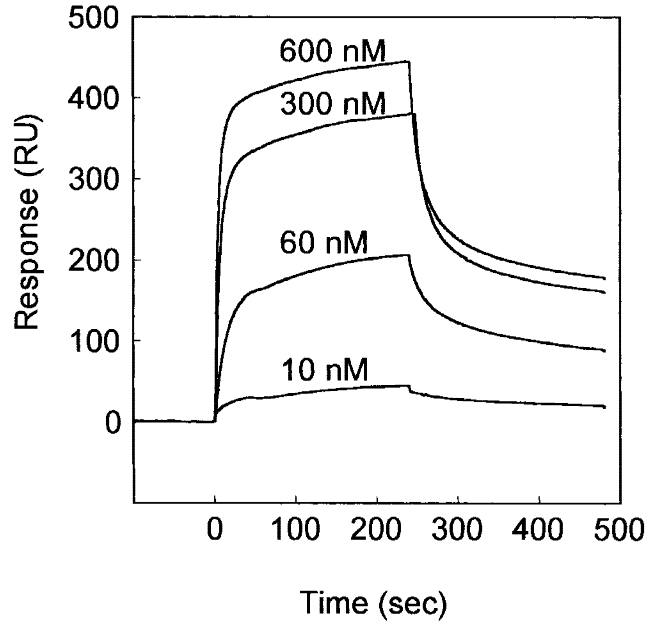

Fig. 4. Surface plasmon resonance analyses of the interactions between rhs-TM and thrombin. rhs-TM solution was injected at time 0 to form rhs-hTM-thrombin complex, and buffer solution was injected after 240 sec to study the dissociation of the complex.

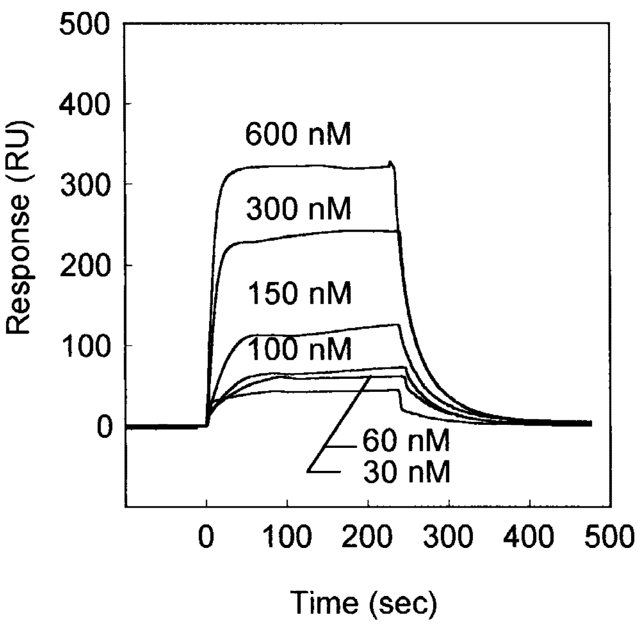

Fig. 3. Surface plasmon resonance analyses of the interactions between f-hTM and thrombin. f-TM solution was injected at time 0 to form f-hTM-thrombin complex, and buffer solution was injected after 240 sec to study the dissociation of the complex.

TM, binding experiments were done after the preadsorption of rhs-TM.

The sensorgrams for f-hTM or rhs-TM are shown in Figs. 3 and 4. Concentrations of f-hTM or rhs-TM (between 10 and 600 nM) were injected randomly, and the binding curves were recorded and k_a, k_d, and K_D were calculated from these data. The fitting calculation of f-hTM was done using a homogeneous model[1]; however, the rhs-TM binding curve could not be analyzed by use of this simple model. From Fig. 3 it appears that the association and the dissociation steps of rhs-TM with thrombin consisted of at least two kinds of interaction phases; here, the kinetic constants of rhs-TM were calculated as a two-phase interaction[2].

Table I summarizes the kinetic constants of the interaction of f-hTM or rhs-TM and thrombin. From the curve

[1]The homogeneous single-site interaction model (A + B = AB, AB = A + B) was used for analyzing f-hTM interaction. Association: $R = R_{eq}(1 - e^{-(k_aC+k_d)(t-t_0)})$. Dissociation: $R = R_0e^{-k_d(t-t_0)}$. Variables: t, time; t_0, start time for the association and dissociation, respectively; k_a, association rate constant; k_d, dissociation rate constant; R_{eq}, steady-state response level; R_0, response at the start of dissociation; C, molar concentration of analyte.

[2]The heterogeneous ligand, parallel reaction model was used for analyzing rhs-TM interaction. This model describes an interaction between one analyte and two independent ligands (A + B1 = AB1, AB1 = A + B1 and A + B2 = AB2, AB2 = A + B2). Association: $R = R_{eq1}(1 - e^{-(k_{a1}C+k_{d1})(t-t_0)}) + R_{eq2}(1 - e^{-(k_{a2}C+k_{d2})(t-t_0)})$. Dissociation: $R = R_1e^{-k_{d1}(t-t_0)} + (R_0 - R_1)e^{-k_{d2}(t-t_0)}$. Variables: t, time; t_0, start time for the association and dissociation, respectively; k_{ai}, association rate constant for component I; k_{di}, dissociation rate constant for component i; R_0, total response at the start of dissociation; R_1, initial response level for component 1; R_{eqi}, steady-state response level for component i; C, molar concentration of analyte.

TABLE I. Kinetic Parameters for the f-hTM or rhs-TM Interactions With Thrombin Using Surface Plasmon Resonance

	k_a (M^{-1} sec^{-1})	k_d (sec^{-1})	K_D (nM)
f-hTM	1.9×10^5	3.9×10^{-2}	205
rhs-TM[a] (slow interaction)	6.3×10^4	4.2×10^{-4}	6.7
(fast interaction)	6.4×10^5	4.8×10^{-2}	75
rhs-TM (Type I)[b]	–	–	26

[a]Type II: thrombomodulin.
[b]By enzymatic method, from [10].

fitting calculation, it became apparent that there were two kinds of interactions (fast interaction with low affinity (K_D = 75 nM) and slow interaction with high affinity (K_D = 6.7 nM)) for rhs-TM with thrombin. The K_D of f-hTM (205 nM) was more than two times higher than the K_D of fast interaction of rhs-TM (75 nM). This shows that f-hTM has a lower affinity to thrombin than does rhs-TM. Comparing K_D of rhs-TM with that of rhs-TM (Type I) (26 nM [10]), it is clear that the rhs-TM used in this study (Type II) had a lower affinity than that of rhs-TM (Type I). The reason for this appears to be due to the differences in the measuring methods (SPR method for rhs-TM (Type II) and the contemporary enzymatic method for rhs-hTM (Type I)) or different type of hTM. Another possible reason is that rhs-TM (Type II) has different sugar groups from rhs-TM (Type I). Further study is necessary in order to clarify this.

DISCUSSION

The results of this study were significant from the perspective of using f-hTM in the development of antithrombogenic biomaterials. Research has found that one of the major roles of the immobilized hTM (rhs-TM) in regard to identifying high levels of antithrombogenicity can be attributed to the entrapment of thrombin. In a previous study, the authors found that f-hTM was immobilized eight times as much as rhs-TMs onto the poly-(acrylic acid)-grafted surface [21]; therefore, f-hTM could exhibit a high level of anticoagulation activity when it is used while being immobilized on the materials, although this study shows that f-hTM is less able to entrap thrombin than is rhs-TM.

Figure 3 shows that the low affinity to thrombin of f-hTM was due to fast dissociation kinetics. It means that f-hTM forms the complex with thrombin. As one mechanism of antithrombogenic activity of hTM is the activation of protein C via hTM-thrombin complex, the f-hTM–thrombin complex also has the ability to activate protein C [21]. The rapid dissociation rate of the f-hTM-thrombin complex, however, limits the use of f-hTM clinically. For the immobilized f-hTM system (for example, an artificial blood vessel), as thrombin and protein C are supplied by blood, frequent exchange of the blood phase is necessary for the complex of immobilized f-hTM and thrombin to show a high level of anticoagulation activity by activating protein C. In other words, the immobilized f-hTM should not be used in static blood-contacting environments, such as a blood bag, but in dynamic environments such as catheters, blood vessels, and extra-corporeal circuits.

hTM has two binding sites to thrombin: one is an EGF-like domain, and the other is chondroitin sulfate domain [22,23]. For rhs-TM, the binding phase seems to be a two-phase one. As the f-hTM used in this study has no chondroitin sulfate moiety, one of the reasons for the lower binding of f-hTM to thrombin seemed to be the lack of chondroitin sulfate moiety in f-hTM. The two-phase interaction phenomenon of rhs-TM appears to be due to those two binding sites (the EGF-like domain and the chondroitin sulfate).

CONCLUSION

The kinetic constants of f-hTM for interacting with thrombin were comparable to that of rhs-TM. It is clear that both the association and dissociation rates of the interaction of f-hTM and thrombin were very rapid. The use of f-hTM in the development of a novel antithrombogenic biomaterial via immobilization is of value to researchers; however, it should be used in a dynamic environment in the blood (i.e., thrombin-rich environment).

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